[redacted]NT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
9872WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 99/00782	17/12/1999	22/12/1998
Applicant		
DSM N.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   PROCESS FOR THE PREPARATION OF OPTICALLY ACTIVE ALPHA-AMINONITRILES

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
P[redacted] 99/00782

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/00 C12P41/00 C12N9/80

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 108, no. 13, 28 March 1988 (1988-03-28) Columbus, Ohio, US; abstract no. 108672, KANG, SHINWON ET AL: "Resolution of amino acids. XVII. Effective acyl groups on the hydrolysi of acylamino acids by mold and hog kidney acylases" XP002104184 abstract & MEM. FAC. SCI., KYUSHU UNIV., SER. C (1987), 16(1), 61-8 CODEN: MFKCAL;ISSN: 0085-2635, ---	1,2
A	EP 0 416 282 A (DEGUSSA) 13 March 1991 (1991-03-13) abstract page 2, line 27 --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2000	13/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lejeune, R

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

[redacted]

International Application No
P[redacted] 99/00782

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BECKER A ET AL: "Structure of peptide deformylase and identifiation of the substrate binding site" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 19, 8 May 1998 (1998-05-08), pages 11413-11416, XP002104183 cited in the application page 11413, column 2, paragraph 2	6

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/[redacted] 99/00782

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0416282 A	13-03-1991	DE 3929570 A	07-03-1991
		AT 111517 T	15-09-1994
		CA 2024622 A	07-03-1991
		DE 59007121 D	20-10-1994
		DK 416282 T	17-10-1994
		ES 2058702 T	01-11-1994
		JP 1848979 C	07-06-1994
		JP 3175984 A	31-07-1991
		JP 5063155 B	09-09-1993
		US 5120652 A	09-06-1992
		US 5219741 A	15-06-1993

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ENT COOPERATION TREA

REC'D 11 OCT 2000
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9872WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL99/00782	17/12/1999	22/12/1998
International Patent Classification (IPC) or national classification and IPC C12P13/00		
Applicant DSM N.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/05/2000	09.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer McDonald, C Telephone No. +49 89 2399 2905

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00782

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-15 as originally filed

Claims, No.:

1-12 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-12
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-12
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following document:

D1: BECKER A ET AL: 'Structure of peptide deformylase and identification of the substrate binding site' JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 19, 8 May 1998 (1998-05-08), pages 11413-11416, XP002104183 cited in the application

1) **Novelty - Art. 33 (1) and (2) PCT:**

Claims 1-12 are new, as a process for the preparation of an α-aminonitrile with enhanced optical purity by selectively deformylating one enantiomer of a chiral N-formyl α-aminonitrile from an enantiomeric mixture by contacting the said mixture with an acylase, is not known from the available prior art. Claims 1-12 therefore fulfil the requirements of Article 33(2) PCT.

2) **Inventive Step - Art. 33 (1) and (3) PCT:**

Document D1, which is considered to represent the most relevant state of the art, discloses the use of peptide deformylase with Fe^{2+} ions for the removal of formyl groups at the end of polypeptide chains in eubacteria, from which the subject-matter of Claim 1 differs in that the deformylation reaction carried out by the peptide deformylase is being used towards the preparation of enantiomerically pure α-aminonitrile.

The problem to be solved by the present invention may therefore be regarded as the provision of a method for the preparation of enantiomerically pure α-aminonitrile.

That the use of peptide deformylase, by the selective deformylation of one enantiomer of an enantiomeric mixture of a chiral N-formyl α-aminonitrile, can be applied towards the preparation of enantiomerically pure α-aminonitrile, is neither disclosed nor suggested in the prior art, and it would not be an obvious process for

the man skilled in the art to utilise the peptide deformylase in such a manner in order to solve the problem posed. It is considered as involving an inventive step. The subject-matter of Claims 1-12 therefore fulfils the requirements of Article 33(3) PCT.

Re Item VIII

Certain observations on the international application

If the terms "Crownpak", mentioned in the description on p. 11 lines 21 and 25, and "Nucleosil", mentioned in the description on p. 12 line 14, are registered trademarks or product names, they should be acknowledged as such (PCT Guidelines C-II, chapter 4.16).

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 August 2000 (02.08.00)	
International application No. PCT/NL99/00782	**Applicant's or agent's file reference** 9872WO
International filing date (day/month/year) 17 December 1999 (17.12.99)	**Priority date** (day/month/year) 22 December 1998 (22.12.98)
Applicant QUAEDFLIEG, Peter, Jan, Leonard, Mario et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

23 May 2000 (23.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

P[redacted]ENT COOPERATION TRE[redacted]Y

From the RECEIVING OFFICE

To: Ms. M.S.N. Jacobs
OCTROOIBUREAU DSM
P.O. Box 9
6160 MA Geleen

DSM P&T
18 JAN. 2000
BA | Reg. 1B | Bericht Nr. 030919

PCT

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.5(c))

Date of mailing *(day/month/year)*
17 January 2000 (17.01.2000)

Applicant's or agent's file reference
9872WO

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL99/00782	17 December 1999 (17.12.1999)	22 December 1998 (22.12.1998)

Applicant
DSM N.V. et al.

Title of the invention
Process for the preparation of α -aminonitriles with enhanced optical purity

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:

[X] was transmitted to the International Bureau on 21 january 2000 (21.01.2000)

[ ] has not yet been transmitted to the International Bureau for the reason indicated below and a copy of this notification has been sent to the International Bureau *:

[ ] because the necessary national security clearance has not yet been obtained.

[ ] because *(reason to be specified)*:

* The international Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c))

Name and mailing address of the receiving Office	Authorized officer
Bureau voor de Industriële Eigendom P.O. Box 5820 2280 HV Rijswijk The Netherlands	M. van Bree
Facsimile No. +31703986507	Telephone No. +31703986487

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12P 13/00, 41/00, C12N 9/80

A1

(11) International Publication Number: WO 00/37668

(43) International Publication Date: 29 June 2000 (29.06.00)

(21) International Application Number: PCT/NL99/00782

(22) International Filing Date: 17 December 1999 (17.12.99)

(30) Priority Data:
98204370.5 22 December 1998 (22.12.98) EP

(71) Applicant *(for all designated States except US)*: DSM N.V. [NL/NL]; Het Overloon 1, NL–6411 TE Heerlen (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: QUAEDFLIEG, Peter, Jan, Leonard, Mario [NL/NL]; Kochstraat 6, NL–6164 HB Geleen (NL). SONKE, Theodorus [NL/NL]; Caeciliastraat 15, NL–6143 BK Sittard (NL). WAGNER, Adolf, Fritz, Volker [DE/DE]; Vischerstrasse 25, D–71638 Ludwigsburg (DE). BROXTERMAN, Quirinus, Bernardus [NL/NL]; Gelrestraat 11, NL–6151 JA Sittard (NL). BOESTEN, Wilhelmus, Hubertus, Joseph [NL/NL]; Brountslaan 9, NL–6132 BJ Sittard (NL).

(74) Agent: JACOBS, Monique, Sophie, Nicole; DSM Patents & Trademarks, P.O. Box 9, NL–6160 MA Geleen (NL).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PROCESS FOR THE PREPARATION OF OPTICALLY ACTIVE ALPHA–AMINONITRILES

(57) Abstract

Process for the preparation of an α–aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral N–formyl α–aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N–formylaminonitrile is selectively deformylated into the unprotected corresponding α–aminonitrile, and a process for the preparation of an α–aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of a chiral (unprotected) α–aminonitrile is subjected to a formylation reaction in the presence of an acylase and a formylating agent whereby one of the enantiomers is selectively converted in N–formyl α–aminonitrile. Preferably a peptide deformylase with a bivalent metal ion wherein the metal is chosen from group 5–11 of the periodic system, is used as acylase, for instance a peptide deformylase chosen from the class EC 3.5.2.27 or EC 3.5.1.31. Such peptide deformylases often contain the sequences of (I) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ. The bivalent metal is preferably chosen from the group of Fe, Ni, Mn and Co, in particular Ni or Fe.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
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CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
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CM	Cameroon			**PL**	Poland		
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CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
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DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Intern. ..nal Application No
PCT/NL 99/00782

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/00 C12P41/00 C12N9/80

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 108, no. 13, 28 March 1988 (1988-03-28) Columbus, Ohio, US; abstract no. 108672, KANG, SHINWON ET AL: "Resolution of amino acids. XVII. Effective acyl groups on the hydrolysi of acylamino acids by mold and hog kidney acylases" XP002104184 abstract & MEM. FAC. SCI., KYUSHU UNIV., SER. C (1987), 16(1), 61-8 CODEN: MFKCAL;ISSN: 0085-2635, ---	1,2
A	EP 0 416 282 A (DEGUSSA) 13 March 1991 (1991-03-13) abstract page 2, line 27 ---	1,2

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2000	13/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

1

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/NL 99/00782

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BECKER A ET AL: "Structure of peptide deformylase and identifiation of the substrate binding site" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 19, 8 May 1998 (1998-05-08), pages 11413-11416, XP002104183 cited in the application page 11413, column 2, paragraph 2	6

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/NL 99/00782

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0416282 A	13-03-1991	DE 3929570 A	07-03-1991
		AT 111517 T	15-09-1994
		CA 2024622 A	07-03-1991
		DE 59007121 D	20-10-1994
		DK 416282 T	17-10-1994
		ES 2058702 T	01-11-1994
		JP 1848979 C	07-06-1994
		JP 3175984 A	31-07-1991
		JP 5063155 B	09-09-1993
		US 5120652 A	09-06-1992
		US 5219741 A	15-06-1993

PROCESS FOR THE PREPARATION OF α-AMINONITRILES WITH ENHANCED OPTICAL PURITY

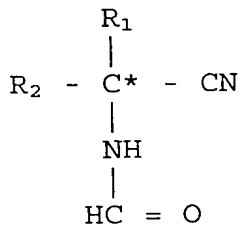
The invention relates to a process for the preparation of an α-aminonitrile with enhanced optical purity wherein a mixture of the enantiomers of the N-formyl-α-aminonitrile is brought into contact with an acylase, whereby one of the enantiomers of the N-formyl α-aminonitrile is selectively deformylated into the unprotected corresponding α-aminonitrile.

There are no processes known in the art wherein a mixture of the enantiomers of an α-aminonitrile is enzymatically, enantioselectively formylated, or wherein a mixture of the enantiomers of an N-formyl-α-aminonitrile is deformylated.

Applicant now has found that it is possible to remove the N-protecting formyl group enantioselectively from one of the enantiomers of a mixture of the enantiomers of N-formyl-α-aminonitriles. In such enantioselective processes moreover very high E-values can be obtained.

α-Aminonitriles to be used as a substrate in the process of the invention are for instance aliphatic and aromatic α-aminonitriles, for example the α-aminonitriles derived from phenylglycine, phenylalanine, *m*-methoxy-phenylalanine, valine and α-methyl-phenylglycine. In the framework of this invention an α-aminonitrile is understood to be an α-aminoacid wherein the carboxy group is replaced by a cyano group.

N-formyl-α-aminonitriles to be used as a substrate are for instance nitriles of formula 1



(1)

wherein:

R_1 represent an, optionally substituted, alkyl or aryl group

R_2 represents H, an, optionally substituted, alkyl or aryl group.

The alkyl groups in R_1 and R_2 may be cyclic or linear or branched chains. The alkyl and aryl groups may be substituted. Suitable substituents are for instance, hydroxy, alkyl, alkoxy, e.g. methoxy, mercapto, alkylmercapto, amino, guanyl, carboxamide, halogen, e.g. chloro, aryl e.g., phenyl and hydroxy phenyl, imidazolyl or indolyl.

In another embodiment of the present invention a mixture of the enantiomers of a (non-protected) α-aminonitrile is subjected to a formylation in the presence of an acylase and a formylating agent, whereby one of the enantiomers is selectively converted into the corresponding N-formyl α-aminonitrile.

Suitable formylating agents are for instance formic acid in case a thermodynamically controlled formylation can be performed, or formic acid esters or amides when the formylation is kinetically controlled. In a thermodynamically controlled formylation the equilibrium is shifted towards the side of the formyl derivative, preferably by precipitation

of the formyl derivative.

Moreover it appeared that, starting from α-aminonitriles, the non-formylated α-aminonitriles relatively rapidly racemise at pH values of higher than 5. In such case the optically active N-formyl aminonitrile can be obtained with an enantiomeric excess of more than 90%, in particular more than 95% and with a yield of more than 90%, in particular more than 95%, calculated with respect to the total amount of (racemic) α-aminonitrile starting product.

Suitable acylases that can be used in the process of the present invention are for instance Penicilline acylases for instance Pen-G or Pen-V acylases, metalloproteases, esterases, deacetylases. Particularly useful enzymes are peptide deformylases.

Peptide deformylases (PDF's) are in general enzymes having formyl methionine peptide deformylase activity. The peptide deformylases to be used according to the invention have a more than 10 times, preferably more than 100 times, in particular more than 1000 times, higher activity towards the N-formyl protected α-aminonitriles compared to the corresponding N-acetyl protected α-amino nitriles. Activity here is defined as the catalytic efficiency (also called: specificity constant) K_{cat}/K_m expressed in M^{-1} sec^{-1}; wherein K_m (expressed in mM) represents the Michaelis constant (this is the substrate concentration at which the reaction rate is 50% of the maximum reaction rate observed) and K_{cat} (expressed in min^{-1}) represents the turnover number. It should be noticed that in the literature also other names are being used instead of the name peptide deformylases; in particular the following names may be mentioned here: formylmethionine

deformylase, N-formylmethionylaminoacyl-tRNA deformylase, N-formyl-L-methionine amidohydrolase, N-formylmethionyl-aminoacyl-tRNA amidohydrolase.

Suitable peptide deformylases to be used in the process according to the invention are peptide deformylases classified as EC 3.5.1.27. Preferably, the enzyme is an enzyme having the activity as described for EC 3.5.1.27 because excellent results are being achieved in the deformylation with such enzymes. It should be noticed that until recently it was believed that the enzyme coded as EC 3.5.1.31 is catalyzing a different reaction. In the meantime, however, it has been shown that the enzymes known as EC 3.5.1.27 and EC 3.5.1.31 are coded for by exactly the same gene and have the same activity. Therefore, as used herein, the term EC 3.5.1.27 is encompassing not only EC 3.5.1.31, but likewise all other enzymes having the same activity as described for EC 3.5.1.27.

Although the family of PDF's is composed of proteins with a relatively low level of sequence identity, the 3D structures of the members of this family appear closely related one to each other with, in particular, the building of a common fold around the bivalent metal ion and three signature sequences. As is described (for PDF's indicated as PDF) by Wagner et al., J. Biol. Chem., **273**, 11413-6 (1998), for many of these enzymes characteristically three short amino acid stretches are present as strictly conserved motifs, namely in that the enzymes contain the sequences (i) HEXXH, (ii) EGCLS and (iii) GXGXAAXQ. In these sequences X represents any natural amino acid, and standard one letter codes for amino acids are used: A = alanine, C = cysteine, E = glutamic acid, G = glycine, H = histidine, L = leucine, S = serine and Q = glutamine.

Peptide deformylases are obtainable for

instance from eubacteria for example *Escherichia coli, Bacillus subtilis, Clostridium acetobutylicum, Clostridium beijerinckii, Haemophilus influenzae, Thermotoga maritima, Thermus aquaticus, Thermus thermophilus, Calothrix PCC 7601, Bacillus stearothermophilus,* or *Lactococcus lactis*. Preferably an enzym obtainable from *Escherichia coli* is used.

Preferably a peptide deformylase is used with a bivalent metal ion whereby the metal is chosen from the groups 5-11 of the periodic system (New IUPAC version; see Handbook of Chemistry and Physics 70th edition, CRC Press, 1989-1990, inner page of cover), as a cofactor. Preferably the metal is chosen from the group of V, Cr, Fe, Ni, Mn, Co, Cu, Pd and Pt, in particular from the group of Fe, Ni, Mn and Co, most preferably Fe or Ni.

Preferably the amount of the bivalent metal ions should be about equivalent to the number of moles of enzyme. Suitably the molar ratio between these bivalent metal ions and the number of PDF molecules is in the range of 0.6 to 1.4, preferably of 0.8 to 1.2, and most preferred the amount of bivalent metal ions is equimolar to the enzyme.

Exchange of the bivalent metal ions in the PDF's in order to obtain PDF enzymes with a co-factor as necessary for the present invention can be done by the various methods as described in Groche *et al.*, Biochem. Biophys. Res. Comm., **246**, 342-346, (1998). These methods include simple metal displacement by incubation of the native enzyme in an excess of the desired bivalent metal ion, if necessary preceeded by

the preparation of the apoenzyme via treatment of the native enzyme with a metal chelation compound. Furthermore, the desired bivalent metal ion can already be introduced in (at least part of the enzyme molecules) by using a bacterial growth medium with an enhanced ratio of the desired bivalent metal ion over Fe^{2+}.

In addition measures may be taken in order to enhance the stability of the enzyme, for instance the addition of stabilisation agents, for instance catalase, tris-(2-carboxyethyl)phosphine, glucose oxidase, or combinations thereof; or enlarging the concentration of the PDF, for instance to a PDF concentration of at least 0.1 mg of PDF per ml, more preferably of at least 1.0 mg/ml. The upper limit of the concentration of PDF is not critical if practical concentrations are being used. The use of stabilisation measures is especially preferred when an easily oxidisable metal ion, e.g. Fe^{++} is present as a cofactor or an easily oxidisable substrate. If not, for instance in case Ni^{++} is present as a cofactor, the addition of a stabilisation agent appeared to be superfluous, as the enzyme turned out to be very stable even without stabilisation agent.

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Alternatively, genetically engineered mutants of PDF's may be used which have for instance enhanced activity or enantioselectivity in the (de)formylation reaction. These mutants can be generated by a number of different approaches; for instance, by site-directed mutagenesis, site-specific random mutagenesis, regio-specific random mutagenesis, and completely random mutagenesis; the latter form of mutagenesis is better known as directed evolution. General applicable methods to perform these different protein engineering approaches are well known to the skilled man. If a random approach will be applied, the mutagenesis cycle will need to be followed by selection of resistent and active mutant(s), thereby leading to the identification of suitable mutants. To obtain PDF mutants also a combination of different protein engineering approaches and/or several rounds of random mutagenesis may be used.

The reaction conditions for the enzymatic deformylation or formylation according to the invention are not very critical and may depend on the substrates

used. Any suitable solvent system which is inert towards the PDF may be applied; such solvents include aqueous systems (solutions or slurries) or aqueous systems also containing a water-miscible organic solvent which is inert under the reaction conditions. Aqueous systems, however, are preferred. Also the concentration of the *N*-formyl compound is not critical, and may be for instance in the range of about 0.1 to 1000 mM. It is not necessary that all of the *N*-formyl compound is dissolved; part of it may be present as a slurry. The concentration of the PDF likewise is not very critical, and usually will be at 0.001 to 100 % by weight of the formyl compound, e.g. at about 0.2 mM of PDF. The pH for the reaction preferably is chosen in the range of 4.0 to 11.0, more preferably of 5.0 to 10.0. The optimum pH is determined by the stability of the α-aminonitrile and/or the N-formyl-α-aminonitrile, and/or the stability and/or activity of the enzyme. The person skilled in the art can easily determine the optimum pH-value. The temperature is not very critical, and suitably will be in the range of 10 to 50°C, e.g. at about 37°C, but for thermostable PDF enzymes higher temperatures may be applied.

In those cases wherein the absolute configuration of the (de)formylated enantiomer was determined, it appeared that the S-enantiomer was (de)formylated more rapidly than the R-enantiomer. The optical purity is given by the enantiomeric excess (ee), the enantioselectivity of the enzyme is represented by E, and calculated as k_f/k_s wherein k_f is

defined as the rate constant of (de)formylation of the most rapidly (de)formylated enantiomer and k_s is defined as the rate constant of (de)formylation of the least rapidly (de)formylated enantiomer.

Optionally a salt promoting hydrophobic interactions is added to the reaction mixture, for instance a sulphate, phosphate, sulphite or acetate of ammonium, Rb, K, Na, Cs or Li. Most preferably ammonium sulphate or lithium sulphate is used.

The invention will further be elucidated by the following 3 examples, without being limited thereto.

Abbreviations:

TB medium: 12 g/l of Bacto-Tryptone, Difco; 24 g/l of yeast extract, Difco; 4 g/l of glycerole; 2.3 g/l of KH_2PO_4; 12.5 g/l of K_2HPO_4);

Hepes: *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphuric acid;

AEBSF: 2-aminoethyl-p-benzene sulphonyl fluoride;

TCEP: tris-(2-carboxyethyl)-phosphine.

MOPS: 3-(N-morpholino) propane sulphonic acid

MES: 2-(N-morpholino) ethane sulphonic acid

Isolation of PDF(Fe)

For a detailed discussion of the methods used reference is made to Groche et al., BBRC **246**, 342-346 (1998).

PDF(Fe) was isolated from overproducing *E.coli* cells grown at 30°C in 1.6 l TB medium for 14-16 h. About 13 g (wet weight) cell paste were suspended in 26 ml buffer (20 mM Hepes/KOH, 100 mM KF, pH 7.7 supplemented with 10 µg/ml catalase from bovine liver (Boehringer Mannheim) and 1 mM AEBSF, disintegrated by sonication (Branson B12, 20 min) at 0°C and centrifuged at 200.000g for 1 h. The clear supernatant (1.3 g of protein; according to biurete reaction) was mixed with 1.3 ml 10%(w/v) Polymin G-35 (BASF) adjusted to pH 7.7 and centrifuged at 40.000g for 10 min. The supernatant was applied to a 20 ml Met-Lys-Sepharose column that had been equilibrated with 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7. After washing with 120 ml of 20 mM Hepes/KOH, 100 mM KF, 0.2 mM TCEP, pH 7.7, PDF(Fe) was eluted with 150 ml 20 mM Hepes/KOH, 100 mM KCl, 0.2 mM TCEP, pH 7.7. The protein containing fractions were concentrated by ultrafiltration using an Amicon PM10 membrane (yield: 140 mg protein, 1400 U/mg; determined according to Groche et al). After adjustment of the TCEP concentration to 1 mM and protein concentration to 40 mg/ml, the PDF(Fe) stock solution (40 mg/ml = 2 mM) was stored frozen at -60°C.

After thawing, the PDF(Fe) stock solution could be used directly in the deformylation experiments described below. If however solutions with lower PDF(Fe) concentrations were required for these deformylation experiments, the PDF stock solution was diluted further in 20 mM Hepes/KOH, pH 7.7, 100 mM KCl, 1 mg/ml bovine serum albumin, 10 µg/ml catalase solution.

HPLC-analysis

In all cases HPLC conditions had to be developed in which the two deformylated isomers were separated from each other and from the formylated isomers. To this end two different techniques were applied, i.e. method A and method B, as described below.

From the concentrations of deformylated isomers in the samples after various reaction times, the (de)formylation rate constant (k_f and k_s in $M^{-1}s^{-1}$) could be calculated for both enantiomers, as well as the respective ee values of the deformylated product. The enantioselectivity of the enzyme (E value) was calculated by taking the ratio k_f/k_s and is given, as well as the maximum ee value of the deformylated product observed during the experiments, in the examples below.

Method A (without derivatization)

A Crownpak CR(+) column (4x150 mm) was used. Samples (5 µl) withdrawn from the deformylation mixture were mixed with 95 µl aqueous $HClO_4$ (10 mM) to inactivate PDF(Fe^{2+}). Following a brief centrifugation, 20 µl of the supernatant were applied to the Crownpak CR(+) column. For specific chromatographic conditions and retention times see the examples II and III.

Method B (Precolumn derivatization with o-Phthaldialdehyde (OPA) and N-acetyl-L-cysteine;

(NAC))

Samples (25 µl) withdrawn from the deformylation mixture were mixed with 25 µl aqueous $HClO_4$ (100 mM) to inactivate PDF (Fe^{2+}). Following a brief centrifugation, 40 µl of the supernatant were added to 80 µl 1 M aqueous H_3BO_3/NaOH pH 11, and subsequently 20 µl OPA reagent (consisting of OPA in H_2O/CH_3OH 1:1 v/v with a concentration as indicated in the example) was added, and 10 minutes later 20 µl NAC reagent (consisting of NAC in H_2O/CH_3OH 1:1 v/v with a concentration as indicated in the example) was added. After 5 min derivatization was terminated by addition of 80 µl (500 mM) aqueous H_3PO_4, and 20 µl of the solution were instantaneously applied to a Nucleosil 120-5 C_{18} column (250 x 4 mm). Temperature is ambient and detection is spectrophotometric using a wavelength of 257 nm and/or 340 nm; the used eluent is a mixture of 80 mol% aqueous 0.05 M H_3PO_4 (brought at pH = 7.0 with 1 M NaOH) and 20 vol% CH_3CN.

Example I: Deformylation of *N*-formyl-valine aminonitrile in the presence of Li_2SO_4 at pH = 7.2.

The deformylation reaction of *N*-formyl-valine aminonitrile was performed in a 1.5 ml Eppendorf reaction test tube. The reaction mixture with a total volume of 200 µl contained 100 mM aqueous MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2, and 10 mM of *N*-formyl-valine aminonitrile. After thermal equilibration to 37°C the deformylation reaction was started by the addition of

50 µM of PDF. At various reaction times samples of the reaction mixture were withdrawn in which the reaction was stopped by addition of $HClO_4$.

HPLC-analysis was performed according to method B, with [OPA] = 16 mg/ml, and [NAC] = 4 mg/ml, retention times: 8.6 min (L-enantiomeer), 10.2 min (D-enantiomeer).

Results:

E = 47.9

ee_{max} = 95.5

k_s = 0.62 $M^{-1}s^{-1}$

k_f = 29.7 $M^{-1}s^{-1}$

Example II: Deformylation of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile without Li_2SO_4 at pH 7.2

The deformylation reaction of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile was performed as described in example I, with the exception that 100 mM MOPS/NaOH, 250 mM NaCl, 0.1 mg/ml catalase buffer pH 7.2 was used instead of 100 mM MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2. Futhermore, 7.2 mM of *N*-formyl-*m*-methoxy-phenylalanine aminonitrile and 2.5 µM of PDF were used.

HPLC-analysis was performed according to method A
Eluent: 90 vol% 10 mM aqueous $HClO_4$/10 vol% CH_3OH
Flow rate: 0.8 ml/min, temperature: 5° C, detection: 210 nm,
retention times:

Deformylated enantiomer(s): 23.8 min.
30.7 min.
N-formyl aminonitrile: 52.0 min.

Results:

E = 685

ee_{max} = 99.0

k_f = 1370 $M^{-1}s^{-1}$

k_s = 2 $M^{-1}s^{-1}$

Example III: Deformylation of *N*-formyl-phenylalanine aminonitrile without Li_2SO_4 addition at pH 6.2.

The deformylation reaction of *N*-formyl-phenylalanine aminonitrile was performed as described in example I, with the exception that 100 mM MES/NaOH buffer pH 6.2 was used instead of 100 mM MOPS/NaOH, 2 M Li_2SO_4 buffer pH 7.2. Furthermore, 7.5 mM of *N*-formyl-phenylalanine aminonitrile and 20 µM of PDF were used.

HPLC-analysis was performed according to method A
Eluent: 90 vol% 10 mM aqueous $HClO_4$/10 vol% CH_3OH
Flow rate: 0.8 ml/min, temperature: 5° C, detection: 210 nm,
retention time:
deformylated aminonitrile: 11.8 min
15.1 min
N-formyl aminonitrile: 28.6 min.

Results:

E = 880

$ee_{max} = 98.8$

$k_f = 880$

$k_s = 1$